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Award Number: W81XWH-05-1-0305

TITLE: Role of Cdc25C Phosphatases in Human Breast Cancer

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REPORT DATE: May 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2006	Annual	8 Apr 2005 – 7 Apr 2006

4. TITLE AND SUBTITLE

Role of Cdc25C Phosphatases in Human Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0305

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mount Sinai School of Medicine
New York, NY 10029-6574

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES - Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

A summary is presented of research performed during the first year of a project to determine the role of Cdc25 phosphatases in human breast cancer. Three specific aims are being pursued. The first is to determine the role of Cdc25B in breast cancer proliferation. The second aim is examining whether alternative splicing of Cdc25C contributes to human breast cancer. The final aim is to explore a potential novel breast cancer therapy involving altered expression of Cdc25C. The long term goals of this research is to validate a clear role for Cdc25B in breast tumor cell proliferation and to rigorously determine whether Cdc25C may contribute to human breast tumorigenesis in other ways besides its overexpression.

15. SUBJECT TERMS
Cdc25, phosphatase, cell cycle, splicing

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

The Cdc25 family of phosphatases plays key roles in cell cycle progression. Two members of this family, Cdc25B and Cdc25C function in a similar manner and have been implicated in regulating entry into mitosis via removal of inhibitory phosphorylations on the Cdc2 kinase (Draetta and Eckstein, 1997). A role for Cdc25B, but not Cdc25C, has been suggested in human cancer (Kristjansdottir and Rudolph, 2004). Nevertheless, Cdc25C has not been rigorously excluded as contributing to human cancer. It is hypothesized that both Cdc25B and Cdc25C contribute to human breast tumor development. Thus, the focus of this research is to validate a role for Cdc25B in regulating breast tumor cell proliferation and to explore other ways that Cdc25C may contribute to the oncogenic phenotype beside overexpression. With this in mind, three specific aims are being pursued. Cdc25B has been reported to be overexpressed in a variety of human tumor types. In the first aim, the levels of expression of Cdc25B will be examined in human breast tumor cell lines. Levels of expression of Cdc25B will be manipulated in these cell lines to determine effects on proliferation. Cell culture models will be employed to examine the role of Cdc25B in transformation. A role for overexpression of Cdc25C in human cancer has not been found in previous studies. It is hypothesized that there are other mechanisms involving Cdc25C, one of which may be regulation by alternative splicing. In the second aim, the various splice forms of Cdc25C will be characterized and their role in regulating cell growth will be examined. In addition, breast tumor cell lines will be screened for alterations in Cdc25C splicing. Overexpression of Cdc25C has been shown to sensitize cells to DNA damaging agents. p53 downregulates Cdc25C expression in response to such agents, and this repression requires the cooperation of another cellular factor (St. Clair et al., 2004). It is suggested that by abrogating the ability of p53 to repress Cdc25C expression in response to particular chemotherapeutic agents, it will be possible to induce a cytotoxic response. It is hypothesized that by blocking the expression of the cooperating factor, such a therapeutically desirable outcome may be achieved. The focus of this aim is to identify and characterize such a factor as well as perform “proof of principle” studies to validate this overall approach.

Body

Task 1. Screen breast tumor cell lines for levels of cdc25B and cdc25C expression (Months 1-4)

Eight breast tumor cell lines were chosen for study in comparison to the non-transformed but immortalized MCF10A breast epithelial cells. Three of these tumor lines express a wild-type p53 while the remaining nine have sustained missense mutations as shown in Table 1. Protein extracts have been prepared from multiple breast tumor cell lines and immunoblotting analysis was performed (Figure 1). Levels of p53 expression correlated with the known p53 status of these cells. Thus, cell lines without p53 mutation expressed low levels of the protein whereas the mutant p53 was expressed, as expected, at substantially higher levels. The basal level of Cdc25C varied, but also correlated with p53 status. Thus, cells with wild-typ53 p53 expressed low levels of Cdc25C whereas those that had sustained p53 mutation expressed high amounts of Cdc25C. This is consistent with previous studies from out laboratory that p53 transcriptionally represses Cdc25C gene expression (St. Clair et al., 2004). This data argues however that p53 status is the major determinant for Cdc25C protein expression. Mdm2 is a negative regulator of p53 that has been shown to, in turn, be transcriptionally upregulated by p53. Examination of levels of Mdm2 expression in these various cell lines showed that in contrast to Cdc25C, there was no strong correlation between Mdm2 expression and p53 status (Figure 1). However, our findings are consistent with a recently published study showing that a single nucleotide polymorphism in the Mdm2 gene (SNP309) was an important determinant of Mdm2 levels in breast cells that also express estrogen receptor (Hu et al., 2007). Thus, T47D and MCF7 cells show high levels of Mdm2 expression while ZR-75-1 cells have reduced expression (Figure 1), correlating with the SNP309 status in these cells (Table 1). Taken together, this argues that levels of Cdc25C expression, in contrast to another p53 target, Mdm2, is strictly determined by p53 status.

Table 1. Characteristics of breast cell lines

	MCF7	MDA-MB-231	MDA-MB-361	MDA-MB-468	ZR-75-1	SKBr3	T47D
p53	wild-type	R280K	wild-type	R273H	wild-type	R175H	L194F
Estrogen receptor	+	-	+	-	+	-	+
Progesterone receptor	+	-	+	-	+	-	+
Mdm2 SNP309	T/G				T/T		G/G

The levels of Cdc25B is consistently higher in all tumor lines as compared to the non-transformed MCF10A cells and this is independent of p53 status (Figure 1). Thus, these eight tumor lines are suitable for further study of the role of Cdc25B overexpression in their proliferation and tumorigenic properties. Examination of transcript levels using an RT-PCR approach shows that protein levels reflect amounts of Cdc25C and Cdc25B messenger RNA in these cells (Data not shown). These findings support the idea that Cdc25B is overexpressed in human breast cancer and is likely to be an important determinant of tumorgenicity. Higher levels of Cdc25C that are found in mutant p53-expressing tumor lines may also contribute to the neoplastic properties of those cells and needs to be further explored.

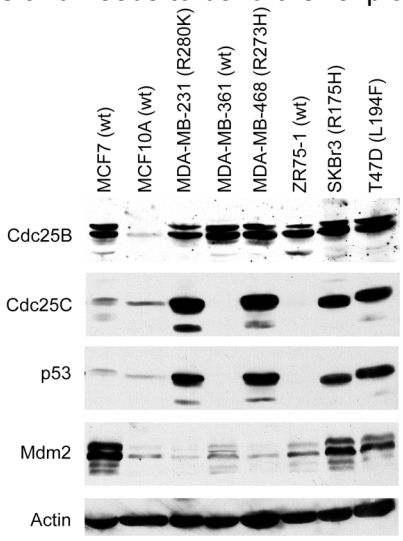

Figure 1. Expression levels of Cdc25B and Cdc25C in multiple breast tumor cell lines.
The indicated cell lines were lysed and subjected to immunoblotting for the indicated proteins. Actin is included as a loading control.

Task 2. Abrogate cdc25B expression in overexpressing breast tumor cell lines (Months 5-12)

To determine the relevance of high levels of Cdc25B expression for the proliferation of breast tumor cells, an siRNA approach was designed to ablate expression of Cdc25B. As the levels of Cdc25C were also found to be high in a subset of the breast cancer lines that express mutant p53 (Figure 1), a strategy for downrgeulation of Cdc25C was also attempted. The siRNA approaches to ablate expression of either Cdc25B or Cdc25C were successful as determined by immunoblotting for protein expression (Figure 2). Interestingly, loss of Cdc25B expression resulted in an alteration in the cell cycle profile of these cells whereas downregulation of Cdc25C had no effect (Figure 2). Although it was gratifying that both siRNA strategies were successful, it was found that loss of Cdc25B expression affected cell proliferation regardless of their transformed phenotype (Data not shown). This suggests that under these conditions it has an important role in regulating normal cell cycle progression. As ablation of Cdc25B affects cell proliferation regardless of their initial levels of Cdc25B expression, the approaches that were optimized here will be uninformative for determining the significance of elevated Cdc25B in the growth of the breast cancer cell lines identified in Task 1. Methods to reduce Cdc25B levels to that seen in MCF10A cells rather than complete ablation of Cdc25B expression may be one way to address this. Strategies to address this are currently being pursued. One possibility is to establish cells with inducible expression of the siRNA in a system in which levels of induction can be tightly regulated. This has been shown to be feasible using tetracycline regulated expression and will be attempted in the future.

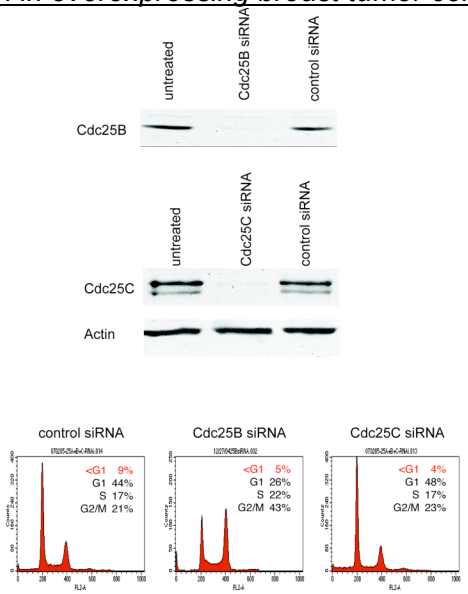

Figure 2. Ablation of Cdc25B but not Cdc25C expression affects cell cycle progression.
MCF10A cells were transfected with either control siRNA oligonucleotides or those directed towards either Cdc25B or Cdc25C as indicated. Cell lysates were then immunoblotted for the indicated proteins. Corresponding dishes of cells were subjected to propidium iodide staining and flow cytometric analysis. Percent of cells with the indicated DNA content were then calculated.

Task 3. Compare ability of cdc25B and cdc25C to cooperate with activated ras in transformation of mammary epithelial cells (Months 13-26)
This task was proposed to be performed in a future funding year.

Task 4. Screen breast tumor cell lines for alternatively spliced forms of cdc25C (Months 1-6)
Preliminary data was presented within the grant application to show that MCF7 and ZR-75-1 cells express two isoforms of Cdc25C. It was thus proposed that alterations in Cdc25C isoforms expression may contribute to breast tumorigenesis. Additional analysis has shown that the relative expression pattern of these two isoforms is unaffected by the tumorigenic phenotype of the cells being examined. Thus, non-transformed MCF-10A cells have a similar expression pattern as the breast tumor lines (Data not shown). These findings do not support one of the hypotheses being tested here, namely that alterations in the relative expression of various Cdc25C isoforms contributes to breast cell transformation. Thus, enthusiasm for further characterization of individual splice forms is reduced, but will be pursued in Task 5 in future funding years.

Task 5. Determine the role of individual splice forms of cdc25C (Months 10-30)
This task was proposed to be performed in a future funding year.

Task 6. Confirm effect of cdc25C overexpression in human breast tumor cells (Months 16-36)
This task was proposed to be performed in a future funding year.

Task 7. Perform "proof of principle" studies (Months 8-24)
Four cdc25C promoter-driven expression plasmids have been generated and are illustrated schematically in Figure 3. Previous studies have shown that enforced expression of Cdc25c abrogates the p53-dependent checkpoints in response to DNA damaging chemotherapy (St. Clair et al., 2004). It was hypothesized that if the p53-dependent repression of Cdc25C could be ablated, that the cellular response could be similarly converted from a cell cycle arrest to an apoptotic outcome. As a "proof of principle" it was proposed to drive expression of Cdc25C in breast tumor cells in a manner independent of p53. The long-term goal is to identify the factor which interacts with the GC box, inhibit its activity, and thereby prevent Cdc25C downregulation. To determine the feasibility of this approach before the factor is actually identified (the goal of Task 8), promoters that drive Cdc25C expression that lack the binding site for this putative factor have been generated (ΔGC). During the design of this initial construct, it was realized that p53-dependent repression via the CDE/CHR element may make interpretation of the results difficult. Thus, the activity of this element was also ablated in a matched set of constructs by scrambling four bases within the CHR. In the next funding year, these constructs will be characterized further and stable clones of particular breast cancer cell lines will be established.

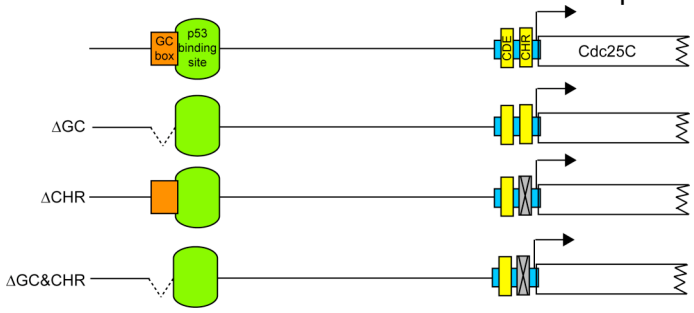

Figure 3. Generation of expression plasmids that drive expression of Cdc25C under control of the human Cdc25C promoter.
The sequence of the human Cdc25C promoter from -225 to +1 was inserted upstream of a cDNA expressing human Cdc25C. Three derivative of this plasmid were then generated. In ΔGC, the eight bases upstream of the p53 binding site (-163/-156) were deleted. In ΔCHR, four bases within the CHR element were scrambled. In ΔGC&CHR, both of these alterations were incorporated in the same construct.

Task 8. Identify factor involved in p53-dependent repression (Months 1-36)
To screen candidate factors for a role in p53-dependent repression, we have utilized an siRNA approach. Sp1 and Sp3 expression have been ablated individually using transient transfection with siRNA oligonucleotides which effectively downregulate their levels. Neither of these showed any effect on p53-dependent repression of cdc25C (Figure 4). We have had difficulty obtaining suitable immunological reagents to use to detect other candidate factors (notably members of the KLF family of transcription factors). Although siRNA oligonucleotides are available that have been reported to affect expression of several of these (KLF4, KLF5, and KLF6), until we can establish a means to detect expression of endogenous proteins, we are unable to address their role in repression by p53. Thus, we have begun to screen a battery of commercially available antibodies to see which will be useful. We have also come to realize that this candidate approach may be problematic if there is redundancy among GC box-binding proteins in our list of likely candidates. There are

four Sp family members and 12 in the KLF family (Turner and Crossley, 1999). Although ablation of each of these individually is feasible and will be pursued, the possibility of performing combinatorial knock-down of two at a time, for example, may become technically difficult. Given these concerns, we are now focusing on the proposed biochemical purification approach to identify novel factors with the hope of validation using an siRNA approach. To facilitate this analysis, the minimal element that is involved in p53-dependent repression needed to be identified. The previous studies had shown that three 10 bp p53 binding repeats were present. Deletion analysis has now shown that loss of the last 10 bp does not affect repression (Figure 5). In the context of this element (-163/-136), mutation of two bases within the adjacent GC box to either TT or AA abrogates repression as was reported for the larger sequence (Figure 5). We now feel that we have identified a minimal element for further study in the biochemical assays proposed.

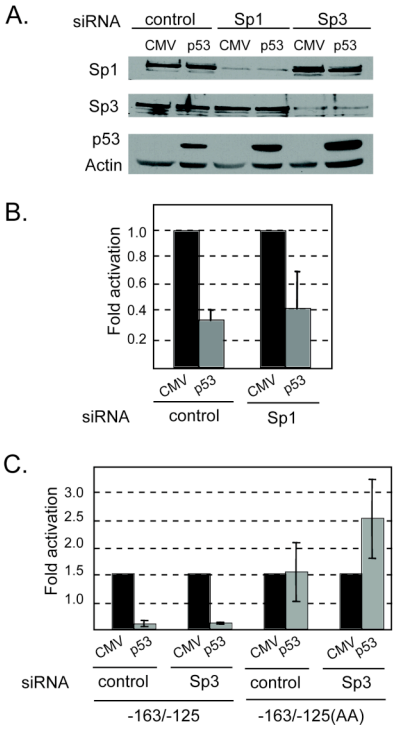

Figure 4. Ablation of either Sp1 or Sp3 does not affect p53-dependent repression of the Cdc25C element.

(A) Cells were transfected with either control siRNA oligonucleotides or those directed against Sp1 or Sp3. Cell lysates were prepared and immunobotted for the indicated proteins. (B-C) Corresponding dishes of cells were co-transfected with either vector alone (CMV) or an expression plasmid for p53 as well as the indicated luciferase reporters. Fold activation was determined compared to the values obtained for each reporter in the presence of vector. Data represents the average of three independent experiments performed in duplicate. Error bars represent standard deviation.

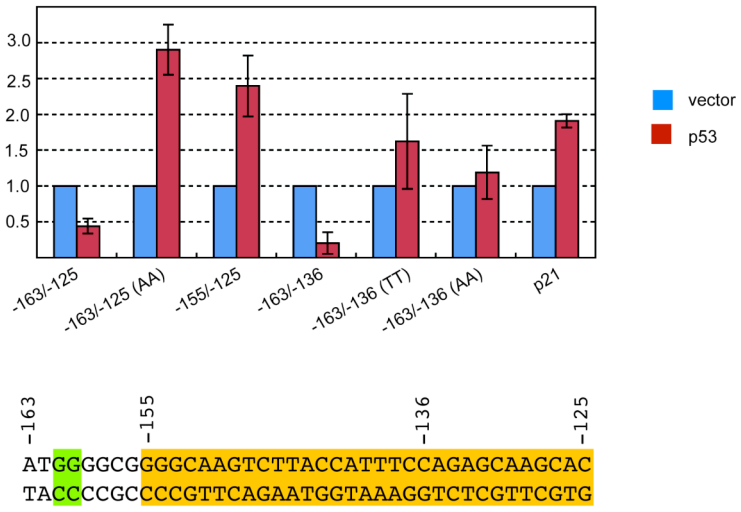

Figure 5. The minimal p53 responsive element for transcriptional downregulation of Cdc25C is contained within -163/-136.

The indicated luciferase reporters were cotransfected with either vector or an expression plasmid for human p53 as indicated. . Fold activation was determined compared to the values obtained for each reporter in the presence of vector. Data represents the average of three independent experiments performed in duplicate. Error bars represent standard deviation.

Key Research Accomplishments

- Demonstrated that Cdc25B is overexpressed in a majority of human breast carcinoma cell lines
- Showed that Cdc25C levels in a panel of human breast carcinoma cell lines correlates with p53 status
- Demonstrated that Cdc25B, but not Cdc25C is required for cell proliferation
- Demonstrated that Sp1 or Sp3 do not contribute to p53-dependent transcriptional repression of Cdc25C
- Determined the minimal element necessary for p53-dependent repression of Cdc25C

Reportable Outcomes

- Established methods for siRNA-mediated ablation of Cdc25B and Cdc25C
- Generated expression plasmids for Cdc25C
- Established methods for siRNA-mediated ablation of Sp1 and Sp3
- Carvajal, L. and Manfredi, J.J. p53-dependent transcriptional repression of human Cdc25C requires a cooperating factor which interacts with an adjacent GC-rich sequence. In preparation.
- Varmeh-Ziaie, S. and Manfredi, J.J. Forced expression of Cdc25C sensitizes tumor cells to DNA damaging chemotherapeutic agents. In preparation.

Conclusions

Although the notion that Cdc25B plays a role in human cancer is not new, the idea that Cdc25C may also be important is an intriguing, novel area of investigation. Further, use of Cdc25C as a tool to design therapeutic strategies to intervene in human breast cancer has been previously unexplored. Much of this specific research is laboratory-based and focuses on feasibility of such approaches. Nevertheless, it represents necessary preliminary studies which will allow further development and translation of these findings in the future with the ultimate goal of establishing a highly effective and targeted therapy for human breast carcinoma.

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Appendices

None.